EXHIBIT 4

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Antagonistic Interplay between Antimitotic and G_1-S Arresting Agents Observed in Experimental Combination Therapy[1]

Korey R. Johnson, Kristy K. Young, and Weimin Fan[2]

Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, South Carolina 29425

ABSTRACT

Paclitaxel is a naturally occurring antimitotic agent that has been shown to stabilize microtubules, induce mitotic arrest, and ultimately induce apoptotic cell death. The favorable clinical activity of paclitaxel has prompted considerable interest in combining paclitaxel with numerous other antineoplastic agents. Our previous studies have suggested 5-fluorouracil (5-FU), an antineoplastic agent that usually arrests tumor cells at the G_1-S phase of the cell cycle, in combination with paclitaxel significantly represses paclitaxel-induced mitotic arrest and apoptosis. In the present study, we have extended this investigation to include several other antimitotic agents (vinblastine, colchicine, and nocodazole) in various combination schedules with the G_1-S arresting agents 5-FU and hydroxyurea (HU). We found 5-FU, as well as HU, could significantly interfere with the overall cytotoxicity as compared with treatment with antimitotic agents alone. It appeared that 5-FU or HU severely limited the antimitotic agents' cytotoxic effects on both mitotic arrest and apoptosis. No combination of a G_1-S arresting agent with an antimitotic agent in any schedule produced an antitumor effect greater than that of the antimitotic agent alone. In addition, biochemical examination revealed that 5-FU and HU blocked the antimitotic agent-induced increase of $p21^{WAF1/CIP1}$ protein levels, as well as prevented the hyperphosphorylation of the bcl-2 and c-raf-1 proteins. These findings suggest that careful considerations may be necessary when combining antineoplastic agents that exert their cytotoxic action at different phases of the cell cycle.

INTRODUCTION

Clinical protocols frequently combine chemotherapeutic agents that exhibit their cytotoxic action at different phases of the cell cycle (1–4). An optimal combination chemotherapy protocol results in increased therapeutic efficacy, decreased host toxicity, and minimal or delayed drug resistance (1, 5, 6). However, when antineoplastic agents with similar or different modes of action are combined, the outcome may be synergistic, additive, or antagonistic. Synergism implies that two drugs may produce greater therapeutic efficacy than an expected additive effect, whereas antagonism implies that the actual therapeutic activity produced by two drugs may be smaller than their expected additive effect (6, 7).

Chemotherapeutic regimes frequently use the novel antimitotic agent paclitaxel. Paclitaxel possesses a unique mechanism of action in that it can bind to microtubulin, stabilize the tubulin polymer, and aid in further polymerization by shifting the dynamic equilibrium toward microtubule assembly (8–12). The microtubules bound by paclitaxel are unusually stable and thereby abrogate the dynamic reorganization process of the microtubule network required to form a functional spindle apparatus for the completion of mitosis and cell proliferation, ultimately leading to programmed cell death (8, 9, 11–17). Paclitaxel has proven especially important clinically because of its activity in the treatment of metastatic breast carcinoma and drug refractory ovarian carcinoma (18–20). The promising clinical activity of paclitaxel has prompted interest in combining this agent with several other very effective antineoplastic agents. One such agent with which paclitaxel is presently being combined in clinical trials is the antimetabolite 5-FU.[3] Specifically, the trials are combining these two agents in various schedules for the treatment of metastatic breast carcinoma, head and neck carcinoma, and gastrointestinal tract carcinomas (21–25). Recently, our laboratory reported that the pretreatment or simultaneous addition of 5-FU (3) with paclitaxel in solid tumor cell lines significantly repressed the overall cytotoxicity as compared with paclitaxel treatment alone (26). It appeared 5-FU interfered with the cell killing activity of paclitaxel by preventing the tumor cells from entering the G_2-M phase of the cell cycle. This finding raises concern as to the effectiveness of combining antimitotic agents with G_1-S arresting agents.

To determine whether this phenomenon applies to the combination of 5-FU with other antimitotic agents, we extended our investigation to include vinblastine, colchicine, and nocodazole. In addition, we combined all four of these antimitotic agents in various regimes with HU, another potent G_1-S phase arresting agent that inhibits ribonucleotide reductase, resulting in depletion of dUMP necessary for DNA synthesis. Our results indicate that the combination of 5-FU or HU with any one of these antimitotic agents produces less antitumor activity than that seen with the treatment of an antimitotic agent alone. These findings suggest that 5-FU and HU may interfere with the

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[2] To whom requests for reprints should be addressed, at Department of Pathology and Laboratory Medicine, Medical University of South Carolina, 171 Ashley Avenue, Charleston, SC 29425; Phone: (843) 792-5108; Fax: (843) 792-7762.

[3] The abbreviations used are: 5-FU, 5-fluorouracil; HU, hydroxyurea; MTT, 3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

cytotoxicity of the antimitotic agents simply by preventing the majority of cells from entering the G_2-M phase of the cell cycle.

MATERIALS AND METHODS

Drugs and Cell Culture. 5-FU, HU, paclitaxel, nocodazole, vinblastine, and colchicine were purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in 100% DMSO to make stock concentrations of 10 mM, 2 M, 1 mM, 1 mg/ml, and 1 mM, respectively. Drugs were then diluted in culture media to obtain the desired concentrations. In all experiments, the various drugs were used at the following concentrations: 10 μM 5-FU, 2 mM HU, 100 nM paclitaxel, 0.2 μg/ml nocodazole, 100 nM vinblastine, and 100 nM colchicine. The human breast cancer Bcap37 (27) and the human epidermoid carcinoma KB cell lines (American Type Culture Collection, Rockville, MD) were propagated in RPMI 1640 supplemented with 10% fetal bovine serum (Sigma) and 1% penicillin/streptomycin. As described earlier (27, 28), the drugs were added when cells reached ~60–70% confluency.

Detection of Internucleosomal DNA Fragmentation. After incubation with the various drug regimes, approximately 1×10^6 cells were harvested and suspended in lysis buffer containing 20 mM Tris, 5 mM EDTA, 0.5% Triton X-100, and 0.5 mg/ml proteinase K for 1 h on ice. The remaining steps for DNA fragmentation were performed as described previously (29). DNA samples were then analyzed by electrophoresis on a 1.2% agarose slab gel containing 0.2 μg/ml ethidium bromide.

Flow Cytometric Analysis. Cell sample preparation and propidium iodide staining were performed according to the methods described by Nicoletti *et al.* (30). At approximately 60–70% confluency, cells were treated with the various drug regimes for 24 and 48 h. Cells were collected and processed as described previously (26). Cell cycle distribution was determined using a Coulter Epics V instrument (Coulter Corp.), with an argon laser set to excite at 488 nm.

Cytospin Preparation. Cells were cultured to ~60–70% confluency, at which time they were treated with the various drug regimes for 24 and 48 h. Cells were harvested by trypsinization and washed twice with PBS. Cell numbers were determined using a hemacytometer, and ~50,000–100,000 cells from each group were used for cytospin preparations. Slides were air dried and fixed in 100% acetone for 5 min prior to Giemsa-Wright staining and then were examined using bright-field microscopy (29).

MTT Assay. Bcap37 and KB cells were harvested with trypsin and resuspended to a final concentration of 4×10^4 cells/ml in fresh medium containing 10% FBS and 1% penicillin/streptomycin. Aliquots of 100 μl from cell suspension were evenly distributed into 96-well tissue culture plates with lids (Falcon, Oxnard, CA). Designated columns were treated for 24, 48, and 72 h. One column from each plate contained medium alone, and another column contained cells in drug-free medium. Cell viability was assessed in accordance to the protocol described by Carmichael *et al.* (31). The absorbancies of individual wells were determined at 560 nm by a microplate reader (Molecular Devices,CA).

Western Blots. Cells were treated with the various drug regimes for 48 h and promptly harvested by trypsinization. Protein extraction and immunoblot procedures were performed as described previously (32). Briefly, the membranes were blocked with BLOTTO for 2 h at room temperature and subsequently probed with 0.5 μg/ml of p53 murine monoclonal antibody (DO-1; Santa Cruz Biotechnology), 0.5 μg/ml of p21 murine monoclonal antibody (WAF-1 AB-1; Calbiochem), 1.0 μg/ml of bcl-2 murine monoclonal antibody (clone # 100; Santa Cruz), or with 0.5 μg/ml of c-raf-1 murine monoclonal antibody (Transduction Laboratories), diluted in 3% BSA-PBST (3% BSA-PBS-0.05% Tween 20). After three 15-min incubations with BLOTTO, 0.1 μg/ml of affinity-purified goat anti-mouse IgG conjugated to horseradish peroxidase (Jackson Immuno-Research) was incubated with the membranes. Immunoreactive bands were then visualized using a chemiluminescence substrate for horseradish peroxidase (Amersham) and exposure to Kodak X-OMAT AR film.

RESULTS

5-FU and HU Interfere with the Capacity of Antimitotic Agents to Induce Apoptotic Cell Death. The hallmark feature indicative of the late stages of programmed cell death is the internucleosomal fragmentation of genomic DNA, which generates a characteristic ladder pattern of ~200-bp intervals when analyzed using agarose gel electrophoresis. Therefore, to investigate the influence of 5-FU and HU on the capacity of the antimitotic agents to induce apoptotic cell death, we simultaneously applied 5-FU or HU with antimitotic agents and compared this to the treatment of the singular agents in the Bcap37 and KB cell lines. Fig. 1 demonstrates the ability of the antimitotic agents paclitaxel, nocodazole, vinblastine, and colchicine to induce DNA fragmentation in the Bcap37 cell line after 72 h of treatment, indicated by the characteristic ladder patterns. Treatment with either 5-FU or HU alone for 72 h was unable to induce DNA fragmentation (Fig. 1, *Lanes 5, 7, 9,* and *11*). However, the simultaneous treatment of 5-FU or HU in combination with paclitaxel, nocodazole, vinblastine, or colchicine for 72 h dramatically reduced DNA fragmentation (Fig. 1, *Lanes 5, 7, 9,* and *11*, respectively). Similar results were seen with the KB cell line (data not shown).

5-FU and HU Hinder the Ability of Antimitotic Agents to Induce G_2-M Arrest. Morphological examination of Bcap37 and KB cell line cultures treated with paclitaxel, nocodazole, vinblastine, and colchicine revealed a significant number of apparent mitotic figures as quickly as 12 h and persisting throughout the 72-h observation. However, the simultaneous addition of 5-FU in combination with the various antimitotic agents significantly decreased the number of apparently mitotically arrested cells (data not shown). Thus, to determine whether 5-FU did significantly interfere with the capacity of the antimitotic agents to arrest cells mitotically, we prepared cytospin slides of both cell lines treated with the antimitotic agents alone or in simultaneous combination with 5-FU. We counted those cells that appeared to contain condensed chromosomes, *i.e.*, mitotic figures, and summarized these results in Table 1. Coincidentally, we found that 5-FU dramatically inhibited the ability of all four antimitotic agents to induce mitotic arrest, as seen in the phase contrast morphological observation. To clarify the exact cell cycle distribution of the Bcap37 cells treated with

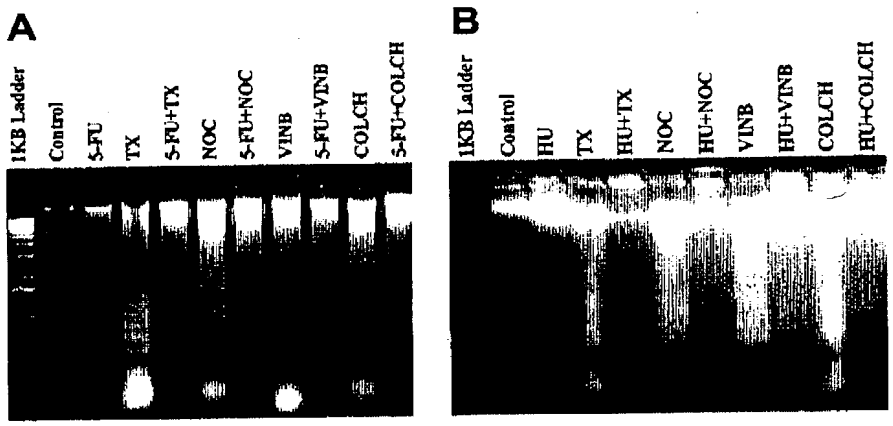

Fig. 1 Effects of the ability of the antimitotic or G_1-S arresting agents to induce internucleosomal DNA fragmentation in Bcap37 cells. Bcap37 cells were cultured in 100 mm tissue culture dishes to ~60–70% confluency. Cells were then treated with either 10 μM 5-FU (*A*) or 2 mM HU (*B*) and were then treated with 100 nM Taxol (*TX*), 0.2 μg/ml nocodazole (*NOC*), 100 nM vinblastine (*VINB*), or 100 nM colchicine (*COLCH*), alone or simultaneously with 5-FU (*A*) or HU (*B*). Total DNA was collected after 72 h exposure to the various drug regimes and electrophoresed on a 1.2% agarose slab gel containing 0.2% ethidium bromide.

Table 1 Effect of 5-FU and antimitotic agents on mitotic arrest[a]

	% of cells at G_2M phase[b]			
	Bcap 37		KB	
Drug exposure	24 h	48 h	24 h	48 h
Control	4 ± 1	5 ± 2	4 ± 2	7 ± 3
5-FU	2 ± 1	1 ± 1	2 ± 1	2 ± 1
Paclitaxel	53 ± 7	62 ± 9	64 ± 7	77 ± 8
5-FU + Paclitaxel	11 ± 6	10 ± 3	15 ± 8	17 ± 5
Nocodazole	51 ± 8	68 ± 11	59 ± 12	81 ± 10
5-FU + Nocodazole	12 ± 8	16 ± 6	16 ± 9	13 ± 7
Vinblastine	60 ± 9	70 ± 8	64 ± 11	73 ± 6
5-FU + Vinblastine	16 ± 8	11 ± 9	18 ± 7	14 ± 7
Colchicine	58 ± 6	66 ± 6	62 ± 9	80 ± 10
5-FU + Colchicine	13 ± 6	15 ± 8	17 ± 7	14 ± 8

[a] This table is based on three separate experiments and presented as mean ± SE.

[b] Cytospin slides were stained with Giemsa. Cells (300) were counted from each slide, and only those cells with typical morphological features of condensed chromosomes were counted as mitotically arrested cells.

the various drug regimes, we performed flow cytometric analysis. Fig. 2 clearly illustrates the capacity of all four antimitotic agents to arrest the majority of cells in the G_2-M phase of the cell cycle. However, pretreatment with 5-FU for 6 h, followed by the addition of antimitotic agents, or even simultaneous addition of 5-FU in combination with the antimitotic agents, dramatically reduced the number of cells in the G_2-M phase of the cell cycle. Interestingly, the pretreatment with the antimitotic agents for 6 h, followed by the addition of 5-FU, partially blocked the number of cells in the G_2-M phase of the cell cycle. Similar results were found when HU was substituted for 5-FU in each protocol (data not shown).

The Cytostatic Effect of 5-FU and HU Interferes with the Cytotoxicity of Paclitaxel in a Schedule-dependent Manner. Previous studies in our laboratory demonstrated that the IC_{50} for 5-FU in the Bcap37 and the KB cell lines was 10 μM. We tested 10 μM 5-FU in combination with IC_{90}s of a variety of antimitotic agents, such as paclitaxel, nocodazole, vinblastine, and colchicine, in various schedules of administration. Each antimitotic agent was incubated simultaneously with 5-FU, and in separate experiments, 5-FU was added 6 h before or after each antimitotic agent. Each antimitotic agent, as well as 5-FU, was applied alone. The drug regimes were carried out for 24, 48, and 72 h. At the end of each time course treatment, the MTT assay was performed as described in "Materials and Methods" to measure cell viability. We found that the pretreatment or simultaneous exposure of the tumor cells with 5-FU could significantly interfere with the cytotoxic effects of all four antimitotic agents (Fig. 3). However, pretreatment of the tumor cells with antimitotic agents could clearly attenuate the inhibitory effect of 5-FU. It should be noted that no combination of 5-FU with an antimitotic agent produced a cell killing efficiency as great as the treatment with the antimitotic agent alone. Similar MTT studies were performed in which HU was combined with all four antimitotic agents in the previously described drug regimes, and data were concurrent with those discussed above (data not shown). Clonogenic survival assays were performed in conjunction with the MTT assays, and these results also correlate with the above data (data not shown).

5-FU and HU Prevent Antimitotic Agent-induced Hyperphosphorylation of bcl-2 and c-raf-1, in Addition to Blocking the Increase in $p21^{WAF1/CIP1}$ Protein Levels. Schandl *et al.* (33) recently demonstrated the ability of the antimitotic agents paclitaxel, nocodazole, vinblastine, and colchicine to induce the hyperphosphorylation of bcl-2. It has been proposed that the hyperphosphorylation of bcl-2 inactivates this protein and coincides with apoptotic cell death (34, 35). Therefore, we investigated the effect of 5-FU or HU on the ability of antimitotic agents to induce hyperphosphorylation of bcl-2 in the Bcap37 and KB cell lines. Fig. 4 (*Lanes 3, 5, 7,* and *9*) clearly demonstrates the capacity of all four antimitotic agents to induce bcl-2 hyperphosphorylation. However, the simultaneous addition of 5-FU or HU with the various antimitotic agents attenuated their ability to hyperphosphorylate bcl-2 (Fig. 4, *Lanes 4, 6, 8,* and *10*).

Blagosklonny *et al.* (36, 37) recently published a study implicating the active phosphorylated form of c-raf-1, repre-

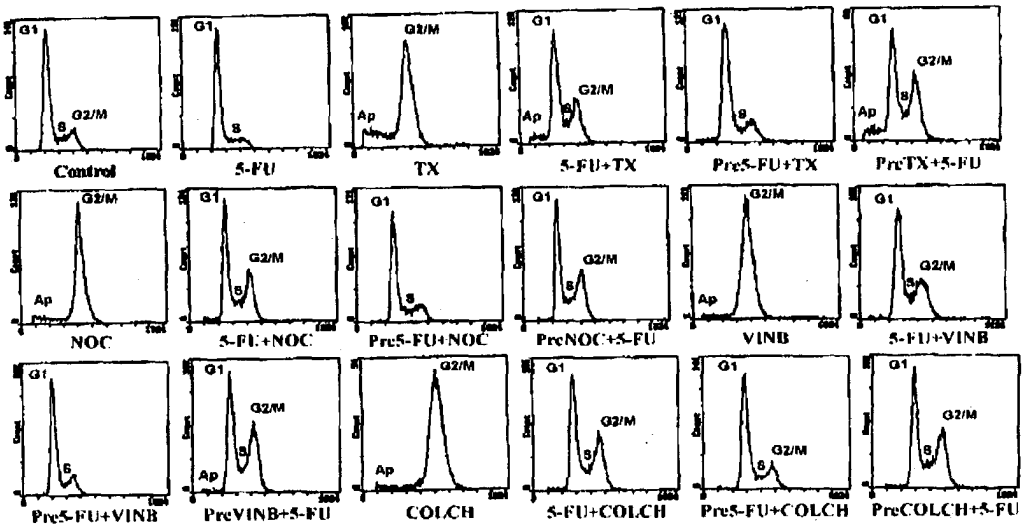

Fig. 2 Flow cytometric analysis depicting the distribution of cells in various phases of the cell cycle. Beap37 cells were treated for 48 h with 10 μM 5-FU, 100 nM paclitaxel, or 100 nM vinblastine (*VINB*), alone or in the combinations indicated. Where applicable, pretreatments were performed for 6 h. Samples were analyzed by flow cytometric analysis as described in "Materials and Methods." The distribution of cells in G_0-G_1, S, and G_2-M phases of the cell cycle and apoptotic cells (*Ap*) are indicated above each corresponding peak. *TX*, Taxol; *NOC*, nocodazole; *COLCH*, colchicine.

sented by decreased electrophoretic mobility on SDS-PAGE, following paclitaxel treatment, to be associated with the hyperphosphorylation of bcl-2 and subsequent induction of apoptotic cell death. Our studies correlate with these findings in that paclitaxel (in addition to nocodazole, vinblastine, and colchicine) induced a slight decrease in electrophoretic mobility of c-raf-1 [previously demonstrated by Blagosklonny *et al.* (39) to be an active phosphorylated form of c-raf-1] in cohorts with bcl-2 hyperphosphorylation (Fig. 4, *Lanes 3, 5, 7,* and *9*). As was seen with bcl-2, simultaneous addition of 5-FU or HU with the antimitotic agents blocked the hyperphosphorylation of c-raf-1 (Fig. 4, *Lanes 4, 6, 8,* and *10*).

In addition, several experiments have indicated that the antimitotic agents paclitaxel, vinblastine, and nocodazole could induce the expression of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ (38, 39). We sought to investigate the possible changes in $p21^{WAF1/CIP1}$ protein levels in the Beap37 and KB cell lines, following the treatments with the various drug regimes. Fig. 4 (*Lanes 3, 5, 7,* and *9*) clearly demonstrates the increase in protein levels of $p21^{WAF1/CIP1}$ after treatment with all four antimitotic agents. Simultaneous treatment with either 5-FU or HU in combination with any one of the antimitotic agents appeared to abrogate this increase in $p21^{WAF1/CIP1}$ protein levels.

DISCUSSION

In this study, we examined the effects of combining chemotherapeutic agents that exhibit their cytotoxic action at different phases of the cell cycle. In particular, we looked into the combination of an antimitotic agent (paclitaxel, nocodazole, vinblastine, or colchicine) with an antimetabolite (5-FU or HU) to determine possible increases in overall cytotoxic effects, as compared with single-agent treatment alone. These combinations are particularly interesting in that paclitaxel is presently being combined with 5-FU and HU in clinical trials (21–23).

Antimitotic agents largely target the microtubule network in individual cells. These agents disrupt the dynamic reorganization of this network, resulting in aberrant mitotic aster formation. Thus, affected cells are unable to transverse successfully from metaphase to anaphase. Ultimately, the prolonged mitotic arrest in most of these cells leads to programmed cell death, or apoptosis. For example, paclitaxel has proven to be especially effective in its cell killing proficiency, apparently through this disruption of the microtubule network (8, 40). Morphological examination revealed that the majority of solid tumor cells treated with clinically relevant concentrations of paclitaxel sustained prolonged mitotic arrest, followed by eventual induction of apoptosis (27, 29, 41 42). In fact, recent evidence from our laboratory indicated that a sustained mitotic arrest is required for paclitaxel-induced apoptosis. When cells were arrested in the G_1-S phase through treatment with 5-FU followed by paclitaxel, we noted a marked decrease in programmed cell death as compared with cells treated with paclitaxel alone. Subsequently, we found no apparent beneficial cytotoxic effects through any *in vitro* schedule combination of 5-FU and paclitaxel (26). The present study demonstrates that the combination of 5-FU with other antimitotic agents, such as vinblastine, colchicine, and nocodazole, once again produces no beneficial cytotoxic effect. Similar experiments were performed on the Beap37 and KB cell lines, in which all four antimitotic agents were combined in a

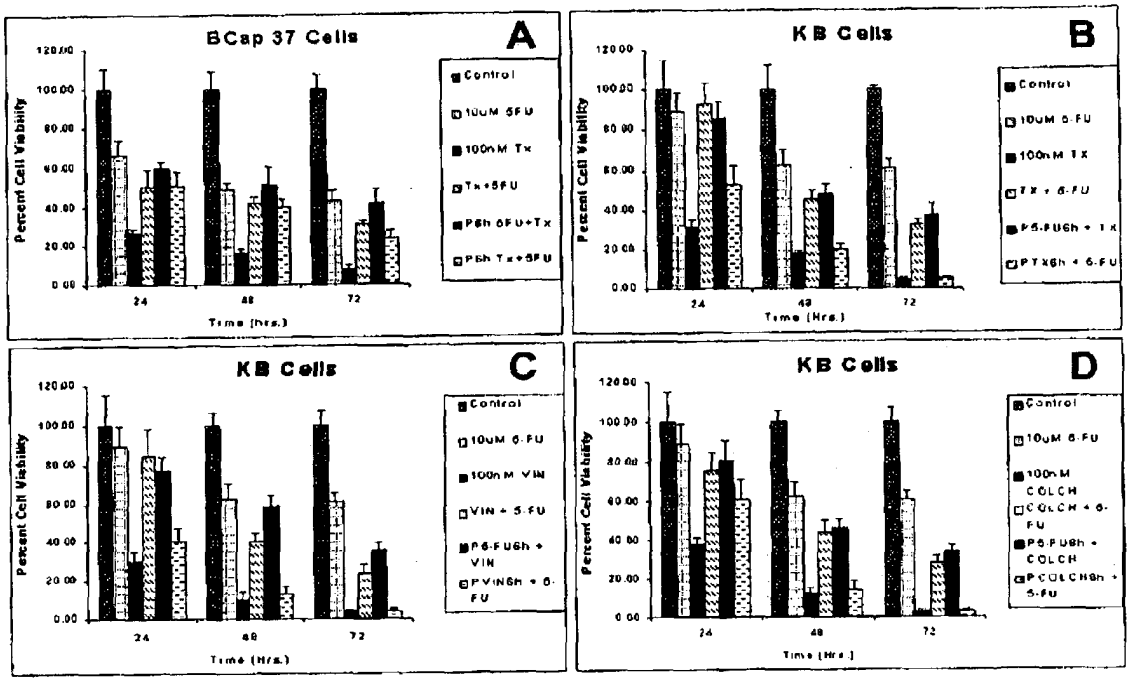

Fig. 3 Cytotoxic effects exhibited by antimitotic and G_1 arresting agents in the Bcap37 and KB cell lines. Approximately 2×10^3 Bcap37 (*A*) and KB (*B–D*) cells were cultured in 96-well microculture plates. Cells were incubated with 10 μM 5-FU, 100 nM paclitaxel (*A* and *B*), 100 nM vinblastine (*C*), or 100 nM colchicine (*D*). Also, incubations were performed in which 5-FU was added simultaneously with paclitaxel (*A* and *B*), vinblastine (*C*), or colchicine (*D*). Furthermore, cells were pretreated with 5-FU for 6 h before the addition of paclitaxel (*A* and *B*), vinblastine (*C*), or colchicine (*D*) for an additional 24, 48, or 72 h. Lastly, cells were pretreated with paclitaxel (*A* and *B*), vinblastine (*C*), or colchicine (*D*) for 6 h before the addition of 5-FU for an additional 24, 48, or 72 h. MTT assays were performed as described in "Materials and Methods." *Bars*, SD.

variety of schedules with HU, another potent G_1-S-phase arresting agent. As was the case with 5-FU in combination with paclitaxel, HU inhibits the capacity of all four antimitotic agents to induce apoptotic cell death (Fig. 1*B*). Flow cytometric analysis and cytospin preparations demonstrate that both 5-FU and HU could arrest the majority of cells in the G_1-S phase of the cell cycle, thereby preventing entry into the G_2-M phase, unless the antimitotic agent was administered prior to the G_1-S arresting agent. These findings correlate with the ability of these G_1-S arresting agents to interfere with the capacity of the antimitotic agents to induce apoptosis (Fig. 1). Hence, it appears that the mechanism by which 5-FU and HU perturbate the cytotoxic effects of the antimitotic agents is merely by preventing the majority of the tumor cells from accumulating in the G_2-M phase of the cell cycle. These data correlate with several previous studies that suggest that the greatest cytotoxic effects of these antimitotic agents is seen when the majority of cells are arrested in mitosis (14, 15, 43).

An important consideration when combining chemotherapeutic agents that possess different mechanisms of action is the schedule in which the drugs are administered. An optimal combination may produce a synergistic effect in which the therapeutic efficacy is greater than the expected additive effect (6, 44). For example, a group of investigators recently discovered that the administration of paclitaxel prior to cisplatin produced a synergistic or additive effect, whereas the reverse sequence resulted in antagonism (45). Furthermore, Kano *et al.* (46) reported that the pretreatment or simultaneous addition of 5-FU with paclitaxel failed to produce a synergistic effect. However, Kano *et al.* (46) discovered an additive effect when paclitaxel was added prior to 5-fluorouracil. Our results from the cytotoxicity assay that examined the combination of the G_1-S arresting agents 5-FU and HU with the antimitotic agents demonstrated the sequential importance of *in vitro* combination drug regimes in the Bcap 37 and KB cell lines. These data suggest that subadditive effects are generated when 5-FU or HU is added prior to or in simultaneous combination with an antimitotic agent. In fact, only when 5-FU or HU was added 6 h after antimitotic agent treatment did we see a cytotoxic effect nearly equivalent to the treatment of the antimitotic agents alone. Thus, the data from this study suggest that the treatment of antimitotic agents alone produced the greatest overall cytotoxic effect in the Bcap37 and KB cell lines.

In light of the fact that the phosphorylation of the bcl-2 oncoprotein has recently been implicated in G_2-M arrest (33) and the induction of apoptosis (34, 35), we performed Western

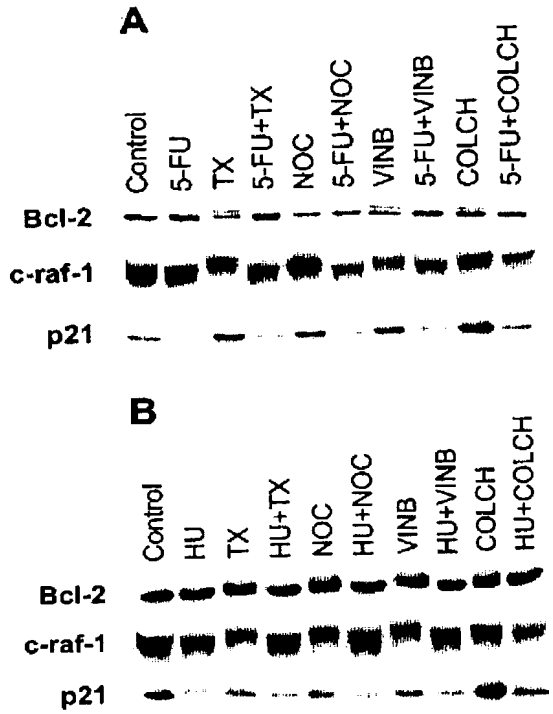

Fig. 4 Western blot analysis for the bcl-2, c-raf-1, and $p21^{WAF1/CIP1}$ proteins. Total whole-cell protein extract was collected from Beap37 cells treated for 48 h with either 10 μM 5-FU (*A*) or 2 mM HU, 100 nM paclitaxel, 0.2 μg/ml nocodazole (*NOC*), 100 nM vinblastine (*VINB*), or 100 nM colchicine (*COLCH*; *B*), alone or in combination with 5-FU or HU. Equal amounts (100 μg/lane) of cellular protein were fractionated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were immunoblotted with a monoclonal antibody to $p21^{WAF1}$ (WAF1 Ab-1; Calbiochem), bcl-2 (clone #100; Santa Cruz), or c-raf-1 (Transduction Laboratories) as described in "Materials and Methods."

blot analysis to determine the phosphorylation state of bcl-2 in each treatment regime. Fig. 4 clearly indicates the ability of the antimitotic agents to hyperphosphorylate bcl-2. However, the simultaneous addition of the G_1-S arresting agents with the antimitotic agents blocked the hyperphosphorylation of bcl-2. Coincidentally, c-raf-1 activation through phosphorylation has been demonstrated after antimitotic agent treatment and has been linked to bcl-2 phosphorylation and subsequent induction of apoptosis (34). Our findings demonstrate the capacity of the G_1-S arresting agents to interfere with the c-raf-1 phosphorylation induced by the antimitotic agents (Fig. 4). The treatments with the G_1-S arresting agents also interfered with the ability of the antimitotic agents to increase the protein levels of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ (Fig. 4). All of these findings correlate with the interference of the G_1-S arresting agents with the capacity of the antimitotic agents to induce apoptotic cell death.

In summary, this study has investigated the possible influence of two G_1-S arresting agents, 5-FU and HU, on the cytotoxic effects of various antimitotic agents on human solid tumor cells *in vitro*. Our results demonstrate that both 5-FU and HU could interfere with the cytotoxic effects of antimitotic agents on mitotic arrest and apoptosis. Meanwhile, we found that the G_1-S arresting agents specifically perturbated the capacity of the antimitotic agents to induce bcl-2 phosphorylation, c-raf-1 activation, and increase in $p21^{WAF1/CIP1}$ protein levels. These data suggest that the G_1-S arresting agents may prevent the majority of cells from progressing to the G_2-M phase of the cell cycle, where antimitotic agents have been shown to exert their greatest cytotoxic effect (40). In light of these findings, careful consideration or experimental evaluation is necessary when combining antineoplastic agents that exert their cytotoxic action at different phases of the cell cycle.

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